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EFFECT OF VARIOUS INHIBITORS ON TREHALASE ACTIVITY IN ASPERGILLUS NIGER

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ABSTRACT

The black-spored fungus *Aspergillus niger* produces trehalase, which catalyzes the conversion of trehalose to glucose. Conidia from 15-day old culture were used for trehalase extraction, purification and characterization. Enzyme activity was assayed by estimating glucose released using 3,5-Dinitrosalicylic acid. The enzyme was purified 46-fold using Diethylaminoethyl-cellulose column chromatography. Then this purified enzyme was used for inhibition studies using three known trehalase inhibitors, Trehazolin, Validamycin and Validoxylamine A. The results showed that Trehazolin couldn't inhibit trehalase whereas Validamycin and Validoxylamine A showed strong inhibition.

KEYWORDS: *Aspergillus niger*, Trehalase, Inhibition, Trehazolin, Validamycin, Validoxylamine A.

INTRODUCTION

Trehalase (α,α-trehalose-1-C-glucohydrolase,) is a glycoside hydrolase, an enzyme which catalyzes the conversion of trehalose to glucose. Trehalases exist in many prokaryotic and eukaryotic cells including bacteria (Carroll et al., 2007), fungi (Murata et al.,2001), and higher plants (Frison et al., 2007), as well as in insects (Kamei et al., 2011; Shukla et al., 2015) and mammals (Kamiya et al., 2004). Trehalase has been purified and characterized from various organisms such as *Saccharomyces cerevisiae* (Alizadeh and Klionsky, 1996), *Lentinula edodes*, from the seeds of *Cicer arietinum* (Kord et al.,2013).

Only trehalase is responsible for trehalose utilization (Silva et al., 2004; Reguera et al., 2012). Trehalose hydrolysis by trehalase is important in various fungal physiological processes like fungal spore germination and the resumption of growth in resting cells. Fungal cells accumulate trehalose up to 30% of the cell dry mass in response to stressful conditions like heat shock and water loss or during growth restriction due to an imbalance between carbon and nitrogen availability (Lillie and Pringle, 1980; Kandror et al., 2004). Trehalose is also a storage carbohydrate, accumulating under conditions of carbon deficiency in fungi (Parrou et al.,2005). Several *in vivo* experiments have shown that trehalose levels closely correlate with stress resistance. Some *in vitro* experiments have concluded that trehalose is a stabilizing agent of cell membranes and proteins as this sugar could substitute for water to prevent destabilization of lipid bilayers (Simola et al., 2000). Trehalase is also responsible for in vivo growth and virulence of the entomopathogenic fungus, *Metarhizium acridum* (Jin et al., 2015).

Many *Aspergillus* species are known, but *A.fumigatus, A.flavus, A. terreus* and *A. niger* are commonly associated with invasive infection in humans (Saubolle, 2000; Wiederhold et al., 2003). In humans *Aspergillus niger* can cause otomycosis (Nwabuisi and Ologe, 2001). *Aspergillus niger* is also causative agent of a disease called black mould on fruits and vegetables. Thus this study was undertaken to purify and characterize trehalase from *Aspergillus niger.* Trehalose hydrolysis by trehalase is responsible for spore germination in fungal species (Francois and Parrou, 2001), it is likely that by studying the inhibition studies of the enzyme, one may learn to control the germination and thus prevent food spoilage.

MATERIALS AND METHODS

Microorganism and culture conditions

Aspergillus niger NS2 was maintained on 50ml media slants [4% (w/v) oatmeal and 1.8% (w/v) agar] 28 ˚C for 10 days then shifted to 4 ˚C for 5 days.

Purification of Trehalase from *Aspergillus niger*

Conidia from 15 day old culture were harvested. To 5 g spore paste of conidia, 10 g of alumina was added and cell suspension was prepared using 0.01 M sodium citrate buffer containing 1 mM phenylmethanesulfonyl fluoride. This suspension was ruptured using glass beads. The homogenate was centrifuged and resultant paste was suspended in 200 ml of 0.01 M sodium acetate buffer and was centrifuged. To the supernatant solution, 1 M acetic acid was added and the pH was adjusted to 4.0. The precipitate formed was removed by centrifugation. The supernatant was cooled to 0 ˚C, and equal amount of cold acetone was added. The precipitate was collected and dissolved in 10 ml of sodium acetate buffer, and dialyzed against 0.01 M phosphate buffer. The resultant solution adsorbed on Diethylaminoethyl (DEAE)-cellulose column which had been equilibrated with 0.01 M phosphate buffer. The enzyme was eluted by applying gradient of 0.05 to 0.3 M NaCl. All purification steps were carried out at 4 ˚C. At each step of purification, trehalase activity and protein content were determined using Bradford method(1976).

Assay of trehalase

Trehalase activity was assayed using 3,5-Dinitrosalicylic acid method by Miller (1959). The reaction mixture contained 100 mM trehalose, 50 mM sodium acetate buffer (pH 5.0), and 0.25 ml enzyme extract in a final volume of 0.5 ml.

Effect of various inhibitors

Effect of three known trehalase inhibitors, Trehazolin, Validamycin and Validoxylamine A was studied on enzyme activity. Incubation mixtures were prepared containing 50 mM Sodium acetate buffer (pH 5), 6 mM $MnCl_2$, and 50 mM trehalose, all in a final volume of 100 µL. Various amounts of inhibitors were added, trehazolin, varying from 10 to 100 µg, Validamycin ranging from 5 to 80 µg and Validoxylamine A varying from 5 to 100 ng and the reactions were initiated by adding 0.05 mg/ml of purified trehalase to each assay mixture. Tubes were incubated for 15 min at 37˚C, and the amount of glucose produced was determined.

Statistical analysis

The statistical analysis of data was done using paired Student's t-test. P-value ≤ 0.05 were considered significant

RESULTS AND DISCUSSION

To characterize the trehalase activity in *A. niger*, the effect of three known trehalase inhibitors, Trehazolin, Validamycin and Validoxylamine A was studied, on enzyme activity. Trehazolin was added at concentration varying from 10 to 100 µg, there was no effect of the compound on *A. niger* trehalase as shown in Figure 1.

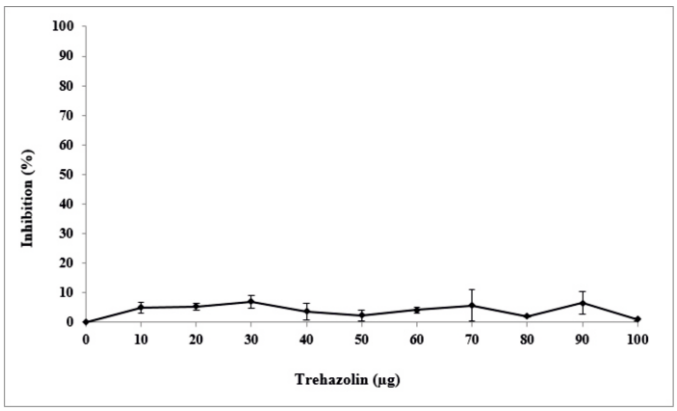

Figure 1: Effect of trehazolin on trehalase activity in *A. niger*. Incubation mixtures were prepared containing 50 mM sodium acetate buffer (pH 5), 6 mM $MnCl_2$, and 50 mM trehalose, all in a final volume of 100 µL. Various amounts of trehazolin, varying from 0 to 100 µg, were then added, and the reactions were initiated by adding 0.05 mg/ml of purified trehalase to each assay mixture. Tubes were incubated for 15 min at 37˚C, and the amount of glucose produced was determined. Data given is the mean of three independent experiments ± standard deviation indicated by bars. **p ≤ 0.005

Effect of Validamycin on trehalase activity was studied by adding varying amount of inhibitor ranging between 5 to 80 µg. These results are presented in Figure 2. Validamycin strongly inhibited the activity of *A. niger* trehalase. There was a gradual decrease in enzyme activity, when concentration of inhibitor was increased. Trehalase activity reduced almost to 10% of the control value at 80 µg of validamycin

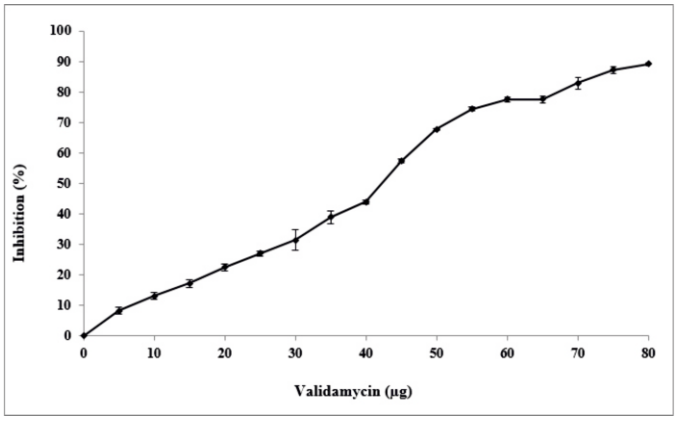

Figure 2: The effect of Validamycin on trehalase activity: Incubation mixtures contained 50 mM Sodium acetate buffer (pH 5), 6 mM $MnCl_2$, and 50 mM trehalose, in a final volume of 100 µL. Various amounts of validamycin, ranging from 0 to 80 µg, was added, and the reaction was initiated by adding 0.05mg/ml of purified trehalase to each assay mixture. Tubes were incubated for 15 min at 37°C, and the amount of glucose produced was determined. Data given is the mean of three independent experiments ± standard deviation indicated by bars. ***$p \leq 0.001$

Further, the effect of Validoxylamine A was also studied. It was added in varying amount between 5 to 100 ng. Validoxylamine A strongly inhibited the *A. niger* trehalase activity. Nearly 86 % inhibition of enzyme activity was observed as shown in Figure 3.

So, among the well known inhibitors of trehalase, only Validamycin and Validoxylamine A inhibited *A. niger* trehalase activity. Relative activity in all these three results is the residual activity left when the inhibitor was added. Its 100 % when no inhibitor was added.

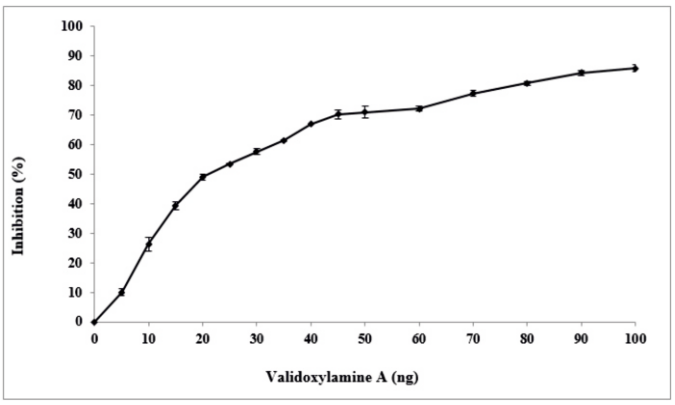

Figure 3: The effect of Validoxylamine on trehalase activity. Incubation mixtures were prepared containing 50 mM Sodium acetate buffer (pH 5), 6 mM $MnCl_2$, and 50 mM trehalose, all in a final volume of 100 µL. Various amounts of Validoxylamie A, ranging from 0 to 100 ng, were then added to these incubations, and the reactions were initiated by adding 0.05 mg/ml of purified trehalase to each assay mixture. Tubes were incubated for 15 min at 37°C, and the amount of glucose produced was determined. Data given is the mean of three independent experiments ± standard deviation indicated by bars. ***$p \leq 0.001$

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